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Introduction

Staphylococcal enterotoxins (SEs) are potential biological threat agents. Exposure to SEs can result in systemic shock and death of military personnel. The enterotoxins, such as SEB, bind as folded proteins to both major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells and germline-encoded variable domain sequences of specific T cell receptor (TCR) beta chain on T lymphocytes. The resulting activation of T cells contributes to several serious diseases and lethal toxic shock syndrome. Rational design of strategies for prevention (vaccines) or treatments of such diseases may only be possible if we have insights into the mechanisms of T cell activation by enterotoxins. Thus, our efforts have been to investigate such mechanisms. Specifically, we are set up to study the molecular interactions of isolated TCR with their natural ligands, peptide/MHC and enterotoxins.

Strategies and procedures

Two individual constructs for bacterial expression of α and β TCR as leucine-zipper fusion proteins were designed. The leucine zipper used here is based on the modified sequence of the naturally occurring leucine-zipper region of Fos and Jun oncoproteins (O'Shea et al., Current Biology, 1993). The α construct, α -ALZ, consist of the genes encoding the α -chain variable and constant domains of Clone1 TCR fused via a linker to the acidic peptide of the leucine zipper. The β -construct, β -BLZ, is made up of the genes encoding the β -chain variable and constant domains of Clone1 TCR fused, through a linker, to the basic peptide of the leucine zipper. In addition the β -construct also has a free unpaired cysteine after the last residue of the basic leucine zipper peptide to facilitate specific immobilization (for BIAcore studies) and fluorescent labeling. The TCR sequences were included up to the cysteine residues involved in the interchain disulfide bridge at the C-terminus of constant domains. A cysteine residue was added to the c-terminus of β chain to facilitate immobilization of TCR in proper orientation for biophysical studies.

The cDNA of α and β T-Cell receptor from Clone 1 T-Cells were constructed using RT-PCR. From the cDNA, the individual constructs for α and β TCR for bacterial expression were made using PCR amplification as follows. The 5' primers were designed to introduce an initiating methionine codon, as part of a NcoI site, just before the first residue of the mature α and β subunits as well as to introduce a XbaI site upstream of NcoI site for cloning purposes. The 3' primers were designed to exclude all the residues following the interchain disulfide as well as to introduce a BamHI site immediately following this cysteine residue. Both such engineered α and β -TCR were cloned into Pbluescript II KS vector as XbaI–BamHI fragments.

The dna coding for the acid/basic leucine zipper and the linker (GSTTAPS) connecting the zipper to the TCR subunits were made using six synthetic oligonucleotides. Briefly, six primers (3 each for the sense and the anti-sense strands), designed to make cohesive BamHI and HindIII sites on either side, were melted and annealed. These annealed double-stranded dna, coding for either the acid-LZ peptide linker or the basic-LZ peptide linker, were then ligated following the BamHI end of pBluescript construct carrying either the α - or β -TCR. Codon usage in *E. coli* was used

as a basis for constructing the dna sequence coding for the leucine-zipper. Furthermore, the unpaired cysteine in the constant domain of β -TCR was mutated to Alanine, to enable more efficient refolding, using Quik Change Mutagenesis technique (Stratagene). The α -ALZ and β -BLZ clones were entirely sequenced to ensure the coding regions had the intended sequence. The NcoI –HindIII fragments of α -ALZ and β -BLZ constructs were then cloned in to pET21-d and pET23-d (Novagen) expression vectors bearing the T7 polymerase promoter. pET21-d has a T7lac promoter contains a lac operator sequence downstream of T7 and offers a more stringent control over uninduced basal protein expression and is typically used to express proteins that may be toxic to bacteria. pET23-d has a plain T7 promoter and works very well with not-so toxic proteins.

Protein expression was attempted in 2 different bacterial strains – BL21(DE3) pLys S and BL21(DE3)-CodonPlus-RIL (Stratagene). BL21(DE3)-CodonPlus-RIL (cells contain extra copies of the genes that code for t-RNAs (Arginine, Isoleucine and Leucine), which most frequently limit translation of heterologous proteins in E.coli.

4 possible combinations in terms of expression vectors and hosts were tried. They were:

- 1) pET21d- based plasmid in BL21(DE3) pLys S cells
- 2) pET23d- based plasmid in BL21(DE3) pLys S cells
- 3) pET21d- based plasmid in BL21(DE3)-CodonPlus-RIL cells.
- 4) pET23- based plasmid in BL21(DE3)-CodonPlus-RIL cells.

As for α goes, there was very little expression observed with BL21(DE3) pLys S cells both with pET21d and pET23d vectors. But noticeable expression was observed with BL21(DE3)-CodonPlus-RIL cells both with pET21d and pET23d vectors. The combination that worked the best so far, in terms of the size of the inclusion-body like pellets, is pET21d- α -ALZ plasmid in BL21(DE3)-CodonPlus-RIL cells. As for the β chain goes, both cell lines and both vectors worked very well. Out of the possible combinations, pET21d-mut β -BLZ plasmid in BL21 (DE3) pLys S and BL21(DE3)-CodonPlus-RIL cells work very well.

The proteins were expressed individually as inclusion bodies in the bacterial strain, BL21(DE3)-CodonPlus-RIL (Stratagene). Inclusion bodies were purified and solubilized in either urea or guanidine. The α and β chains were renatured together by dilution refolding of the denatured protein to form heterodimeric TCR. We followed the refolding procedure described by Garboczi et al first. Later on, we modified his procedure for optimization of refolding.

Beta chain was expressed in inclusion bodies at or > 90% purity, as evaluated by SDS-PAGE and Coomassie staining. However, alpha chain expression was poor and in mixture with many other proteins. Changing the strain of expressing E. coli helped to increase the proportion of the α chain relative to other proteins but did not eliminate expression of contaminating proteins significantly.

Small scale refolding tests were set up to evaluate efficiency of the refolding conditions. We tested different denaturants, changes in pH, presence or absence of

glycerol at varying concentrations (0-30 %), and different ratios of the denatured individual proteins. We continue our efforts in evaluating refolding conditions to find the optimal conditions for TCR folding and large scale purification.

Parallel with our protein purification and refolding efforts, we have been working towards establishing the technology needed for accomplishing specific Aim 2. A novel membrane immobilization strategy for investigating interactions of TCR inserted in cell membrane with the ligand in real time was attempted. Membrane fragments of T cell clones bearing TCR and other proteins involved in T cell activation, such as CD4, were isolated and used for immobilization on the surface of L1 chip of BIAcore. Experiments in progress continue to optimize this technology. This technique can be utilized for measuring interaction of TCR inserted in membranes with superantigens or peptide/MHC ligands in natural environment. Purified TCR immobilized on the surface of BIAcore chip will be compared to the membrane anchored native TCR for accurate determination of the mechanism of interaction. Possibilities of TCR clustering in interaction with MHC/peptide and different superantigens will be evaluated.

Accomplishments):

1. Beta chain of TCR was produced in inclusion bodies of the expressing bacteria to over 90 % purity
2. Alpha chain of TCR was produced in inclusion bodies but mixed with many other proteins
3. At present, we can properly fold TCR from bacteria, as confirmed by ELISA, but with a low yield. We attempted to optimize refolding of TCR by; a) mixing a larger proportion of a chain protein with the beta chain, b) try several protein folding methods, and c) use other strains of bacteria expressing the gene to increase compatibility with the a chain. Adding 5% glycerol helped refolding.

Reference

Erin K. O'Shea, Kevin J. Lumb and Peter S. Kim. Current Biology Peptide 'Velcro': Design of a heterodimeric coiled coil (1993) 3: 658-667.